THE EXTRACTION OF HEMAGGLUTININ FROM STRAINS OF *HEMOPHILUS AEGYPTIUS*[1]

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ABSTRACT

A serologically active substance was extracted from cells of *Hemophilus aegyptius* with dilute NAOH. This substance directly hemagglutinated human erythrocytes. The activity of this material was demonstrated at a near neutral pH and was precipitated at acid pH. Preliminary biochemical determination showed this serologically active material to be predominately polysaccharide.

Davis, Pittman, and Griffiths (1950) reported that strains of *Hemophilus aegyptius* (Koch-Weeks Bacillus) possessed the capacity to hemagglutinate human red blood cells. This property was relatively stable to aging, not lost by frequent transfer of the culture, exposure to low temperatures, or upon treatment with formalin or ether. Erythrocytes treated with the receptor-destroying enzyme of *Vibrio cholerae* were still hemagglutinated by strains of this organism, thereby differing from the influenza and similar viruses. However, the hemagglutinating capacity of this organism was inhibited by antiserum to *H. aegyptius*, prepared in rabbits. It was also determined that this property could not be completely removed from the organism by successive washings with normal saline, and that these washings did not contain appreciable quantities of the hemagglutinin. Supernates or culture filtrates of the organism also possessed this activity, but in quantities less than that observed with intact cells.

The study to be described was initiated to further characterize this property.

MATERIALS AND METHODS

Hemophilus aegyptius strains 18, 46, 128, 145, 180a, 181a, and 763 were generously provided by Dr. Margaret E. Pittman, USDHEW. Cultures were routinely passed and maintained on Brain Heart Infusion medium fortified with five per cent digest of sheep blood (which supplies the necessary X and V factors) after the method of Fildes (1920).

Large quantities of *H. aegyptius* cells were obtained by cultivating the various strains in 32-oz prescription bottles containing the fortified BHI agar. Organisms were harvested after 18 to 24 hr incubation by washing the cells from the surface with unbuffered 0.85 per cent saline. The resulting suspensions were thrice washed in normal saline and tested for the capacity to directly hemagglutinate human erythrocytes.

The extraction of hemagglutinin was accomplished by resuspending the harvested bacterial cells in physiological saline and adjusting the pH to 10 or 11 by the addition of dilute NaOH. This alkalinized suspension was incubated in a 37 C water bath for 30 min and centrifuged lightly for 5 min in a Servall Superspeed Angle Centrifuge. The supernate was recovered and filtered through a millipore filter to insure a bacteria-free solution. The remaining extracted cells were saved for additional serological studies.

A portion of the bacteria-free alkaline extract was adjusted to a pH of 6.5 by the addition of dilute HCl. At this pH, a precipitate formed which was recovered by centrifugation, washed three times in saline, resuspended to the original volume and tested for hemagglutinability. The unadjusted alkaline extract was also tested for this capacity.

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Bacterial cells which had been subjected to alkaline extraction for the removal of hemagglutinin retained to some extent the hemagglutinating property, and therefore were repeatedly re-extracted at a pH range of 10 to 11 in an attempt to remove the remaining serologically active material and also to obtain hemagglutinin-free cells. The resulting cell suspensions were employed for bacterial agglutination studies, hemagglutination tests, and antiserum-adsorption procedures.

All preparations of whole cells, hemagglutinin-free (denuded) cells, and extracts were tested for hemagglutinability. Serial doubling saline dilutions of the test materials were prepared in 0.5-ml quantities. To each tube in the titration series was added 0.5 ml of a 0.5 per cent suspension of washed normal human red cells. The specific type or group of the red cells employed apparently was of no consequence. All tests were incubated in a 37 C water bath for 30 min and then the suspended red cells permitted to settle completely at room temperature. The end point of the titration was considered to be the last tube exhibiting a definite pattern of hemagglutination.

Bacterial hemagglutination-inhibition and extract hemagglutination-inhibition studies were performed using standard doubling saline dilutions of antiserum in 0.25-ml volumes against four units of either the hemagglutinating intact cell or of the active extract contained in 0.25 ml saline. A suspension of 0.5 per cent washed normal human red cells in 0.5-ml quantities was employed as the indicator system. After incubation at 37 C for 30 min, the tubes were allowed to settle at room temperature and read for pattern. The last dilution tube of the series in which there was no evidence of hemagglutination represented the titration end-point.

A suspension of approximately 1×10^9 whole cells per ml served as antigen in bacterial agglutination studies. Tests were incubated at 37 C for 1 hr, refrigerated over-night, and read after light centrifugation. The last dilution presenting evidence of macroscopic agglutination, as compared with a cell-saline control, was considered the end point of the titration.

Antisera against the various strains of *H. aegyptius* were prepared by injecting rabbits with vaccines prepared from washed bacterial cells that had been heat-inactivated for 30 min at 56 C and standardized to a turbidity of 10 billion cells per ml by the addition of saline containing 1:10,000 merthiolate. These vaccines were administered intravenously in 1.0-ml quantities at three day intervals for a nine day period. On the fifteenth day following the first injection, each animal was bled by cardiac puncture, the serum recovered and stored at −4 C until needed.

In an attempt to obtain antibodies specifically directed against the hemagglutinin without interference from extraneous material from the medium or the bacterial cell, normal human red cells were sensitized with the extract and injected as antigens. These antigens were prepared by incubating one volume of washed human erythrocytes with two volumes of the active extract material (pH 6.5) for 30 min in a 37 C water bath. These modified red cells were washed three times in saline to remove unadsorbed material, reconstituted with saline to a volume twice that of the original packed red cells and 1.0 ml injected. After several injections the animals were bled and the recovered antisera were heat-inactivated and repeatedly adsorbed with washed packed human erythrocytes to remove antibodies directed to the red cell carrier. These antisera then were tested for inhibition of both bacterial and extract hemagglutination and for bacterial agglutination.

In the course of this investigation, precipitin ring tests were employed using the alkaline extract (pH 8.5 to 9.0) as the antigen to determine the reactivity of the various antisera. This method was of value for screening purposes, but due to the capacity of this antigen to precipitate at a near neutral pH, the tests were not considered to be as valid as desired. A micro-gel diffusion technique after the method of Yakulis and Heller (1959) was utilized for the detection of antibody.

The adjusted alkaline extract was diffused against antisera to red cells modified with the alkaline extract, and to antisera against intact bacterial cells. These preparations were examined for lines of precipitation after 24 hr.

The capacity of the alkaline *H. aegyptius* extracts to sensitize red cells was investigated. Highly alkaline extracts (pH 10 to 11) as described above were adjusted to pH 8.0 with dilute HCl and the preparation rendered isotonic by the addition of dry NaCl. One tenth ml of washed packed normal erythrocytes and 1.0 ml of the adjusted isotonic extract were mixed and incubated for 30 min in a 37 C water bath after which the excess extract was removed by washing with physiological saline and finally the red cells resuspended to 0.5 per cent by volume. This red cell suspension then was tested for sensitizing capacity of the extract by the addition of antiserum prepared against the whole bacterial cell.

RESULTS

All *H. aegyptius* strains were tested for agglutination by corresponding whole cell antiserum. Titers ranging from 1:128 to 1:1024 were obtained in most cases. By subjecting the intact bacterial cells to repeated alkaline extractions at 37 C for 30 min, it was determined that the ability of these cells to be agglutinated by corresponding whole cell antiserum decreased with each extraction. After six extractions, the extracted cells were essentially inagglutinable by *H. aegyptius* antiserum. These data are summarized in table 1.

TABLE 1

Effect of repeated extraction on agglutinability of H. aegyptius *by specific antiserum*

Serum dilution	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Control
normal cells	4+	4+	4+	4+	3+	1+	+−	−	−	−
4 extractions	2+	1+	+−	−	−	−	−	−	−	−
5 extractions	1+	+−	−	−	−	−	−	−	−	−
6 extractions	+−	−	−	−	−	−	−	−	−	−

Adsorption of the antiserum with previously extracted cells failed to reduce the capacity of the serum to agglutinate the intact, unextracted bacterial cell. Similarly the capacity of *H. aegyptius* strains to hemagglutinate after repeated alkaline extraction was examined and a decrease in this property following successive extractions was noted. The rate of decrease is summarized in table 2.

TABLE 2

Effect of repeated extraction on hemagglutination by H. aegyptius

Bacterial dilution	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Control
normal cells	4+	4+	4+	4+	4+	2+	1+	−	−	−
4 extractions	2+	1+	+−	−	−	−	−	−	−	−
5 extractions	1+	+−	−	−	−	−	−	−	−	−
6 extractions	+−	−	−	−	−	−	−	−	−	−

It also was observed that the crude alkaline extract of *Hemophilus aegyptius* would not consistently produce direct hemagglutination of human red cells and that when this extracted material was acidified with dilute HCl to a pH 6.5 to 6.7, a precipitate occurred. After washing and resuspending this precipitated material in saline, normal human erythrocytes were regularly hemagglutinated, while at a pH of 8.5 to 9.0 the extract failed to demonstrate hemagglutination.

Hemagglutination-inhibition tests utilizing four units of the active extract and antiserum to the whole bacterial cell were achieved. Inhibition of hemagglutination by the active extract was demonstrated with all the anti-bacterial sera tested and to titers reminiscent of those observed with these same sera when tested for agglutinins with whole cells.

Although the crude alkaline extract did not demonstrate hemagglutination directly, passive sensitization of human erythrocytes with this extract was accomplished by adjusting the pH to 8.0 before treatment of the erythrocytes. Agglutination of extract modified red cells occurred again with serial dilutions of antiserum prepared to the intact bacterial cell. Tests were incubated for 1 hr at 37 C (water bath), lightly centrifuged and read for hemagglutination.

Extract-modified red cells also were employed as antigen for the production of antibody directed specifically against the hemagglutinin. The resulting anti-

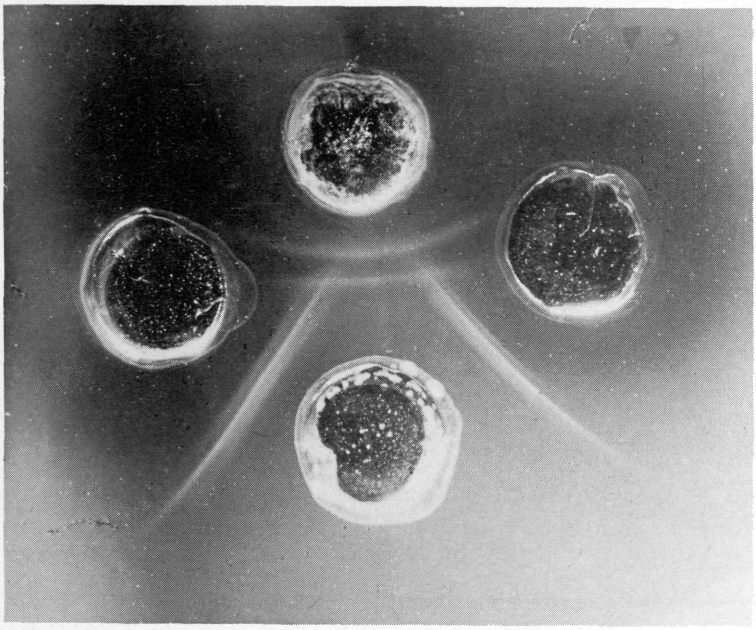

FIGURE 1. Gel Diffusion: Bacterial antisera and anti-extract modified red cell serum against alkaline extract of *Hemophilus aegyptius*.

red cell-extract serum which had been adsorbed with erythrocytes, was found to inhibit hemagglutination by untreated whole bacterial cells and hemagglutination by the active extract to titers of 1:320 to 1:640 respectively.

Using the alkaline extract as a soluble antigen, preliminary studies with agar diffusion technique after the method of Yakulis and Heller (1959), revealed lines of identity when this material was diffused against antiserum to the bacterial cell and against antiserum to the extract modified red cells. In figure 1, three distinct bands between strain 52 bacterial antiserum (left well) and strain 181 antiserum (right well) with the lower antigen well can be detected. Two lines of precipitate were expressed by the antiserum to the alkaline extract modified red cell (top well) and the alkaline extract system (bottom well).

Preliminary biochemical studies showed the active extract to be predominately polysaccharide in composition. However nitrogenous material also was present as determined by Ninhydrin and Biuret procedures.

DISCUSSION

The capacity of *Hemophilus aegyptius* to hemagglutinate normal human erythrocytes was described by Davis, Pittman, and Griffiths (1950). In an attempt to further characterize and define this capacity, a method has been established for the extraction of serologically active hemagglutinin from these cells. This procedure consists of treatment of bacterial cell masses with saline alkalinized with NaOH.

Alkaline-saline extraction has recently been employed by Onoue, Kitagawa, and Yamamura (1963) to recover the endotoxin of *Bordetella pertussis* and is of interest since this organism was at one time included in the genus *Hemophilus*. These workers recovered an extract that was viscous and obtainable at a high pH. Their preparation demonstrated necrotizing activity when administered at a near neutral pH. Similar properties were noted after alkaline treatment of *H. aegyptius* cells; the material was serologically active at a near neutral pH, although it was not tested as a necrotoxin.

It is unusual that the extracted hemagglutinin demonstrated the capacity to sensitize human red cells at pH 8.0, but did not bring about direct hemagglutination. Davis, Crumpton, MacPherson, and Hutchison (1958) have shown that the ability of some polysaccharides to adsorb on to red cells after alkaline treatment may be related to the removal of O-acetyl groups. The adjustment of our extract to pH 6.5 to 6.7 by the addition of dilute HCl resulted in the precipitation of serologically active particles that directly hemagglutinated test erythrocytes, demonstrated inhibitory activity in the presence of anti-serum to the intact bacterial cell and were prevented from hemagglutinating test red cells by antiserum to extract-modified red cells.

Davis, Pittman, and Griffiths (1950) have indicated the effect of hydrogen ion concentration on hemagglutination by *H. aegyptius*. A pH of 6.6 to 6.7 was most favorable while activity was inhibited at pH 8.0 to 8.5. Similar activity was manifested by the active extracted hemagglutinin discussed in this study.

Olitzki and Suliteanu (1958) have employed sonic extracts of non-encapsulated *H. influenzae* and of *H. aegyptius* as antigens in gel diffusions against homologous and heterologous antiserum to demonstrate the presence of antigens common to these often confused organisms. In this study and with alkaline-extracted hemagglutinin from *H. aegyptius*, lines of precipitation were observed between antiserum to intact bacterial cells and with antiserum to red cells sensitized with this hemagglutinin. Precipitin reactions involving alkaline extracted material from *H. influenzae* revealed no cross-reactivity with *anti-H. aegyptius* preparations.

From the preceding, it is suggested that the hemagglutinating factor of *Hemophilus aegyptius* may be, in part, a mucopolysaccharide. This assumption is based on the detection of polysaccharide and hexosamine. Further investigation of the nature of the material extracted from this organism is in process. In conjunction with this study, other extraction methods are being examined in an effort to recover a hemagglutinin from *H. aegyptius* that is relatively free of extraneous or non-functional groups. Preliminary results indicate that the active hemagglutinin is basically carbohydrate with only traces of nitrogen, while the extract discussed in this study is abundant in this substance. Since extracts obtained by the more recent investigation are apparently simpler in composition, exact identification of this material should be facilitated and the results will be the subject of future reports.

SUMMARY

A method is outlined for the extraction by dilute NaOH of substances from cells of *Hemophilus aegyptius* which upon acidification to pH 6.5 to 6.7 will directly hemagglutinate human erythrocytes. This serologically active substance also can be diluted and still demonstrate hemagglutinating activity. Titers observed

are similar to those noted on testing the hemagglutinability of dilutions of standardized, intact *H. aegyptius* cell suspensions.

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